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13. ABSTRACT (Maximum 200 words) We have successfully developed a novel a protein expression system based on the bacterium <i>Ralstonia eutropha</i> . This system has been developed in our laboratory to overcome some of the shortcomings associated with recombinant protein expression in other bacteria (e.g. poor fermentation performance, inclusion body formation and proteolysis). High level expression of organophosphohydrolase (OPH), an enzyme prone to inclusion body formation in <i>Escherichia coli</i> has been demonstrated. A proteomics approach identified the promoter of the <i>phaP</i> gene as a strong, inducible promoter. By creating a translational fusion between the <i>phaP</i> promoter and <i>oph</i> gene and introducing this fusion into the chromosome, OPH titers of greater than 1 g/L were achieved in high cell density fermentations. Multiple copies of the $P_{phaP}::oph$ translational fusion were introduced into the chromosome to further increase expression. A proportional increase in OPH titer was found with increasing copy number. An OPH titer of approximately 4.3 g/L was measured in high cell density fermentation using a strain containing three copies of $P_{phaP}::oph$ translational fusion. This represents the highest titer reported to date for OPH (approximately 30 times greater than previously reported expression levels). Results have reported in two publications (Srinivasan et al. 2003; Srinivasan et al., 2002).				
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Scientific progress and accomplishments:

Introduction:

With advances in protein engineering, there has been a surge in the number of newly discovered proteins and protein function as well as the ability to engineer the properties of such proteins. Enhancement of protein production is a primary goal of recombinant microbial process development. This can be achieved by increasing the amount of recombinant protein per cell (specific productivity) and/or increasing the cell concentration per unit time (cell productivity). In order to improve volumetric productivity in a cost effective manner, recombinant proteins are often produced in high cell density fermentations. High cell density fermentation has many advantages over normal fermentations in that they have a higher final product concentration, reduced waste water and higher overall productivity and hence lower setup and operating costs (Chen, Hwang et al. 1992; Andersson, Strandberg et al. 1994).

We proposed the establishment of a novel enzyme expression system designed around a robust and proven fermentation organism. We have developed the molecular biological research tools required to provide a flexible gene transformation and expression system for *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*), an organism we have chosen based on its unusually robust fermentation characteristics under industrial fermentation conditions. Moreover, we have successfully developed a high cell density fermentation system, to over express recombinant proteins in *R. eutropha*. Organophosphohydrolase was chosen as our model enzyme.

The original project objectives are summarized below.

Objectives:

- (i) Establish a fermentation system based on *R. eutropha* to produce maximum protein titers.
- (ii) Establish transformation and conjugation protocols for introducing genes into *Ralstonia eutropha*.
- (iii) Identify potent transcriptional control elements.
- (iv) Integrate the fermentation and recombinant expression systems.

Results:

1. Fermentation:

We have developed a simple, efficient and reproducible high cell density fermentation protocol based on *R. eutropha* strain NCIMB40124. We have routinely obtained cell densities of over 180g DCW/L in a simple fed batch mode with an industrial medium containing glucose. In order to maximize protein content in the cell, the amount of PHA polymer produced has to be minimized. Previous studies have established that *R. eutropha* produces polymer under phosphate or nitrogen limitations. We performed several experimental fermentations to establish the amount of phosphate that has to be fed for maintaining a high cell density while limiting the polymer content. We were able to demonstrate that feeding the organism with a low carbon: phosphate ratio controlled the PHA concentration. We have established a feeding regime wherein we supply glucose and phosphate during the initial phases in order to increase the cell density to approximately 130 g/L.

Another common problem that occurs in high cell density fermentations is the production of by products such as acetate, lactate and ethanol under oxygen limiting conditions. This has been a major drawback in establishing high cell density fermentations in *E. coli* and *Bacillus*. (Dedhia, Hottiger et al. 1994; Gschaedler, Le et al. 1994; Kleman and Strohl 1994). *R. eutropha* produces polymer instead of these compounds to dissipate its reducing equivalents. In our fermentations the dissolved oxygen concentrations dropped to 0% as soon as cell densities of 30 g/L dry cell weight were achieved and remained at 0% throughout the remainder of the fermentation. We performed HPLC analysis of the fermentation broths at various time intervals during the fermentation. Concentrations of acetate, lactate or ethanol were below the detection limit of 0.1 g/L.

2. Transformation and Conjugation:

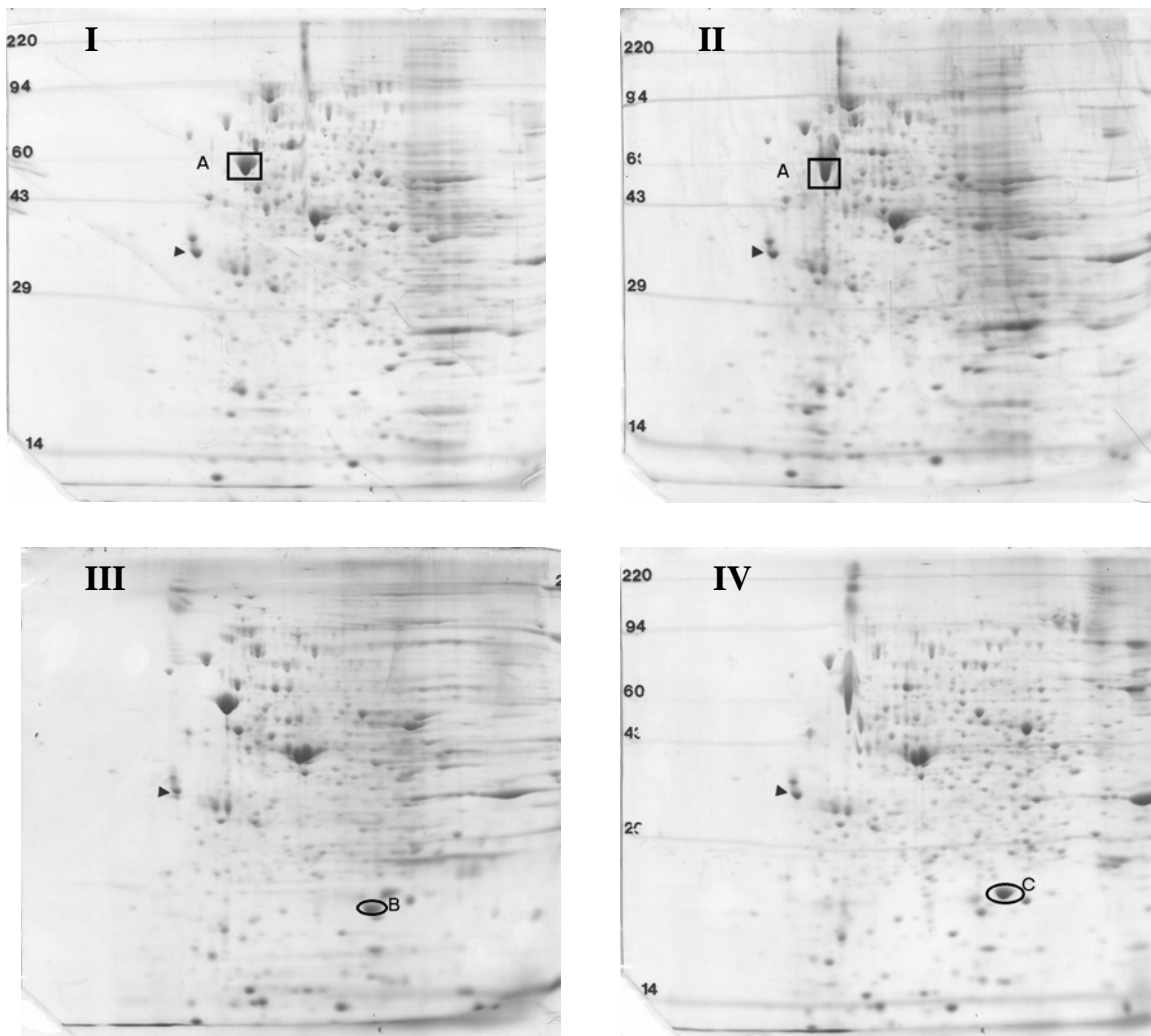
Even though *R. eutropha* grows to very high cell densities, the organism has not been used to produce recombinant proteins. A few genes, most of them involved in PHA production, have been successfully expressed in *R. eutropha* using the pKT230 a broad host range plasmid. (Pries, Steinbuchel et al. 1990). Maintaining the genes in a plasmid offers many advantages such as: (1) manipulation of plasmids is simpler and commonly used in molecular biological applications; (2) plasmids allow us to increase the number of copies (gene dosage) of a given gene thereby increasing expression levels. A major hurdle for establishing transformation procedure in the *R. eutropha* strain is the absence of selectable markers. We found that the strain NCIMB 40124 is resistant to streptomycin and kanamycin, the selectable markers of the plasmid pKT230. We grew the strain on an array of antibiotics and identified that the organism is not resistant to chloramphenicol and tetracycline. We have established the ability to transform recombinant genes into *R. eutropha* by tri-parental mating as described by (Jackson and Srienc 1999) with pKT230, a high copy number plasmid. We have cloned a chloramphenicol acetyltransferase (*cat*) gene into the vector as a selection marker in *R. eutropha*. We have used this technique successfully to transform *R. eutropha* with a plasmid (pKTPPCm) carrying the OPH gene under the control of the *phaP* promoter, chloramphenicol marker and the replication regions of pKT230.

3. Identifying potent transcriptional control elements:

A key element of the project was the search for strong regulatory sequences (promoters) on the chromosome of *R. eutropha*. High expression levels of recombinant proteins can be achieved only if the gene construct for protein expression contains a strong promoter. We were interested in identifying genes that are up regulated during the fermentation process itself or are up regulated in response to the variation of a simple process parameter. Such induction parameters included: (1) a change in temperature; (2) the addition or omission of a specific nutrient e.g., phosphate; (3) micro aerobic conditions; (4) change in pH; or (5) change in carbon source e.g., changing glucose to fructose. We performed fermentations where we altered one of the above parameters and collected samples before and after induction. Of these induction conditions, we were able to achieve high densities (greater than 150 g/L dry cell weight) only for two of the conditions: phosphate limitation and changing glucose to fructose. The total protein from these samples was extracted and separated by 2D SDS PAGE. From the 2D SDS PAGE gels we could identify three strongly expressed proteins: a constitutive protein of about 60 kDa (A); an approximately 20 kDa protein (B) induced by fructose and another protein of about 25 kDa; and (C) a 24 kDa protein induced by phosphate limitation.

The gels were subsequently blotted onto a PVDF membrane and the N-terminus of the proteins was sequenced using Edman degradation. We then searched the GENBANK library for identifying the proteins using BLAST. Protein A showed identity to a chaperonin in *B. cepacia*, while Protein B did not show homology to any other protein. Protein C was identical to a 24 kDa protein from *R. eutropha* named GA24. GA24 is the product of *phaP* gene which binds to the surface of the PHA limiting the size of the polymer (Wieczorek, Steinbuchel et al. 1996). This protein has been found to be present to about 5% of the total protein in the cell under phosphate or nitrogen limiting conditions (i.e., under polymer production conditions). Since phosphate limitation is easy to maintain and polymer production has advantages for high cell density fermentations, we decided to use the promoter of this protein for expression of recombinant proteins. From the sequence of *phaP* gene we synthesized primers for the promoter and cloned it from the genomic DNA of *R. eutropha*.

Fig 1. Two-dimensional electrophoresis of whole cell extracts of *R. eutropha* under fermentation conditions: (I) un-induced at OD₆₀₀ 100, (II) un-induced at OD₆₀₀ 300, (III) fructose induction and (IV) phosphate limitation.



4. Development of the OPH expression system in *R. eutropha*

Our initial strategy to produce OPH was to construct a $P_{phaP}::oph$ translational fusion and introduce this fusion into the cell on a plasmid (pKT230). Good OPH expression results were obtained from shake flasks experiments. However scale-up to high cell density fermentation resulted in very low specific enzyme activities (<1 U/mg protein). Plasmid stability was measured at numerous time points during several fermentation experiments. It was found that only 30% of the cells retain the plasmid, after 20 hours after inoculation and that less than 10% of the cells retained the plasmid after 50 hours.

In order to express OPH in high cell density fermentations of *R. eutropha*, a translational fusion between the *phaP* promoter and the *oph* gene was created and introduced into the suicide plasmid pJQPPCm. This suicide plasmid was used to integrate the recombinant gene into the *phaP* locus of *R. eutropha* using bi-parental mating techniques. Integration into the chromosome overcomes the problems of maintaining plasmid stability over multiple generations during the course of a fermentation experiment.

A Southern blot was performed to confirm that single copies of the $P_{phaP}::oph$ translational fusion had been integrated in the *phaP* locus.

Fig 2: Southern blot analysis of the OPH integrant strain to confirm single copy integration.

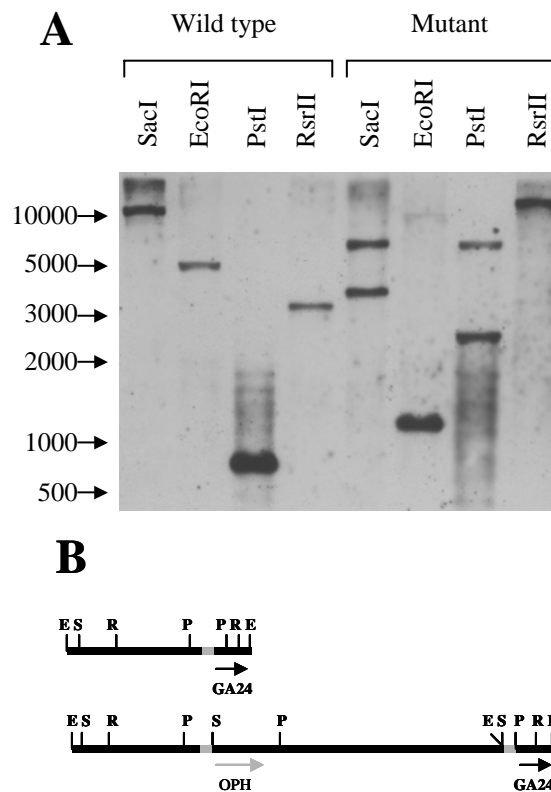


Fig 3: Western blot of fermentation samples from a high cell density fermentation of *R. eutropha* expressing OPH from a single chromosomal copy.

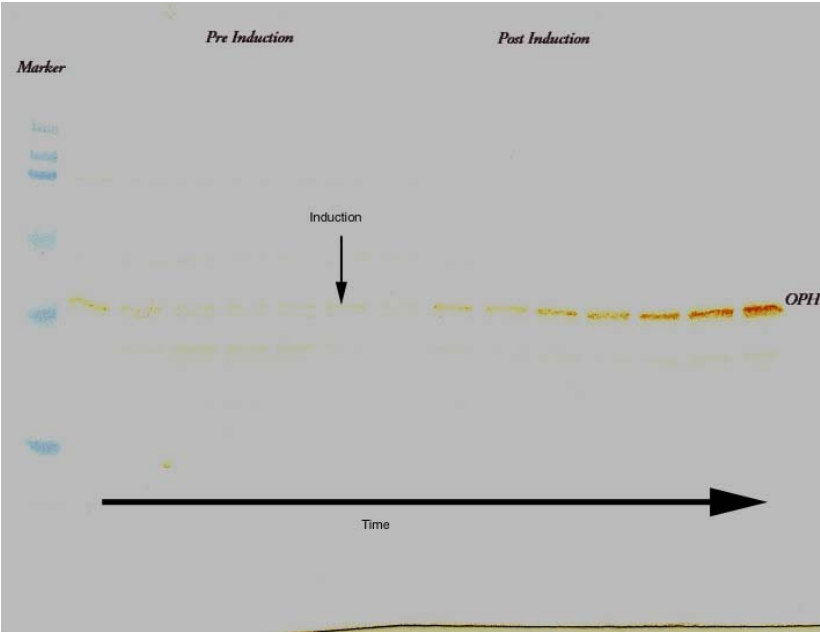


Fig 4: Growth and OPH activity from a high cell density fermentation of *R. eutropha* expressing OPH from a Single Chromosomal Copy.

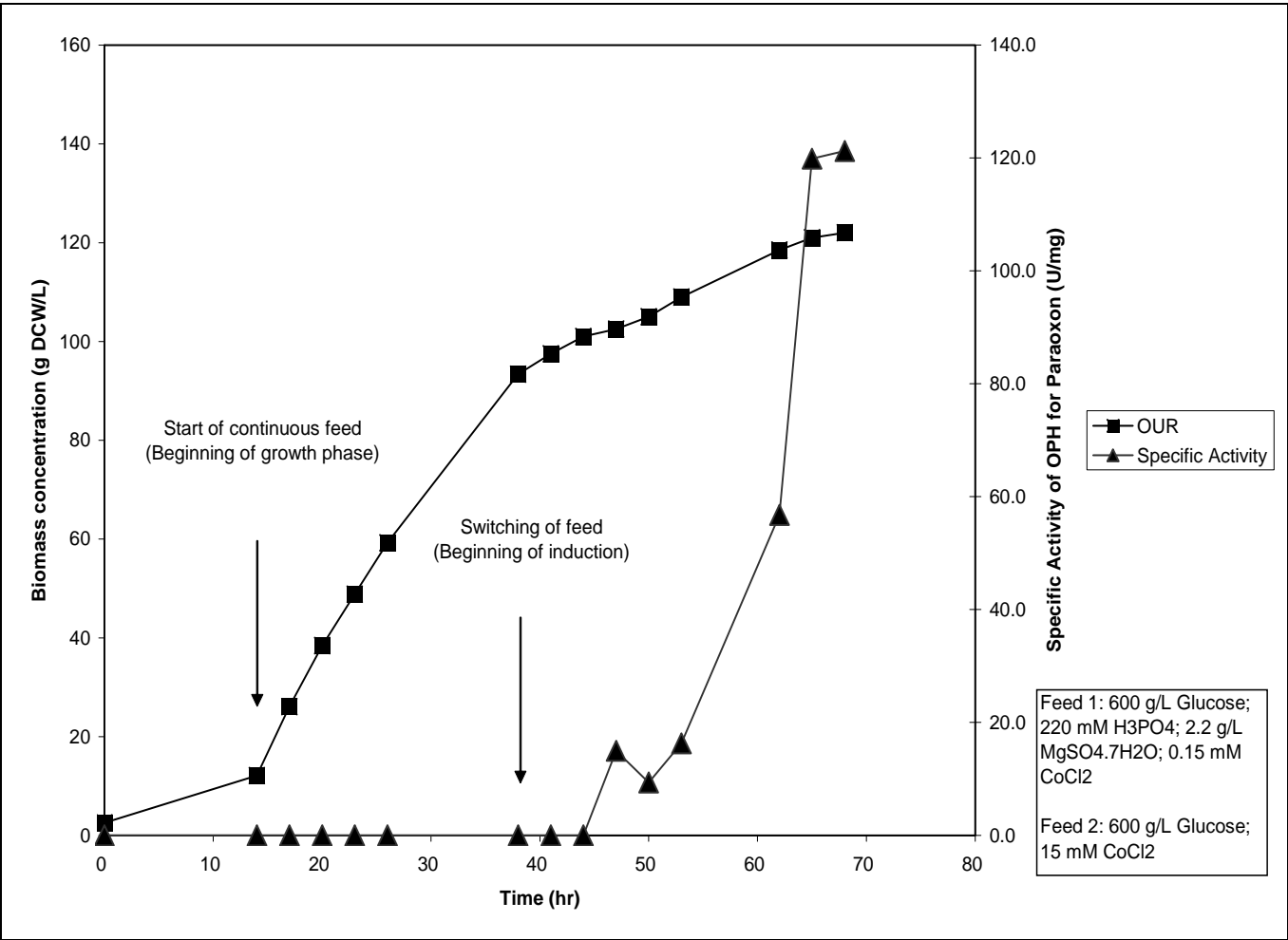


Figure 6 shows high levels of expression (circa. 121 U/mg using the paraoxon assay). This enzyme concentration represents approximately 1.5% of the total protein and thus in this fermentation about 1 g/L of soluble, active OPH was produced. Moreover the Western blot shows that the basal level expression of the protein is low prior to induction with phosphate limitation.

Results from sections 1, 2, 3 and 4 culminated in an Applied and Environmental Microbiology publication (Srinivasan et al., 2002).

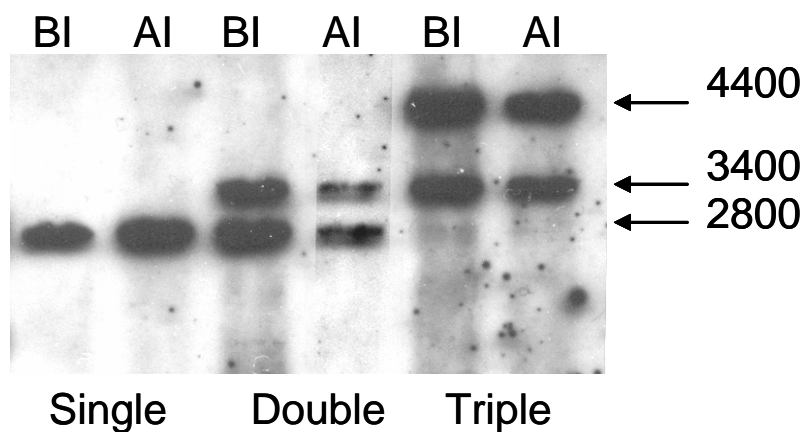
5. Increasing *oph* gene dosage

In prokaryotes, gene dosage has typically been increased by the use of medium and high copy number plasmids. In lower order eukaryotes, gene expression has been achieved random, multiple integration of a gene of interest into the host chromosome (Werten et al., 1999). Multiple copies of the $P_{phaP}::oph$ translational fusion were therefore introduced into the *R. eutropha* chromosome in an attempt to increase gene dosage. Single, double and triple copies of the $P_{phaP}::oph$ translational fusion were introduced into the *R. eutropha* chromosome.

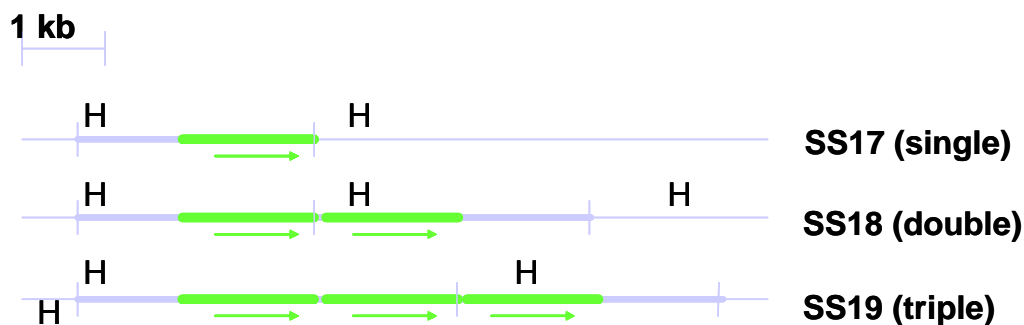
Southern blot analysis showed not only that the multiple copies had been integrated into the chromosome, but also that the integration is stable throughout the time course of the fermentation.

Fig 5: (A) Southern blot analysis of *R. eutropha* strains SS17, SS18 and SS19. Genomic DNA of single copy (SS17; lanes 1 and 2), double copy (SS18; lanes 3 and 4) and triple copy (SS19; lanes 5 and 6) was digested with *Hind*III. Lanes 1, 3, and 5 correspond to samples collected during fermentation before induction. Lanes 2, 4, and 6 correspond to samples collected after induction. (B) Map of the genomic DNA region analyzed. The open reading frame of the *oph* gene is indicated in the figure while H refers to the *Hind*III restriction sites.

A



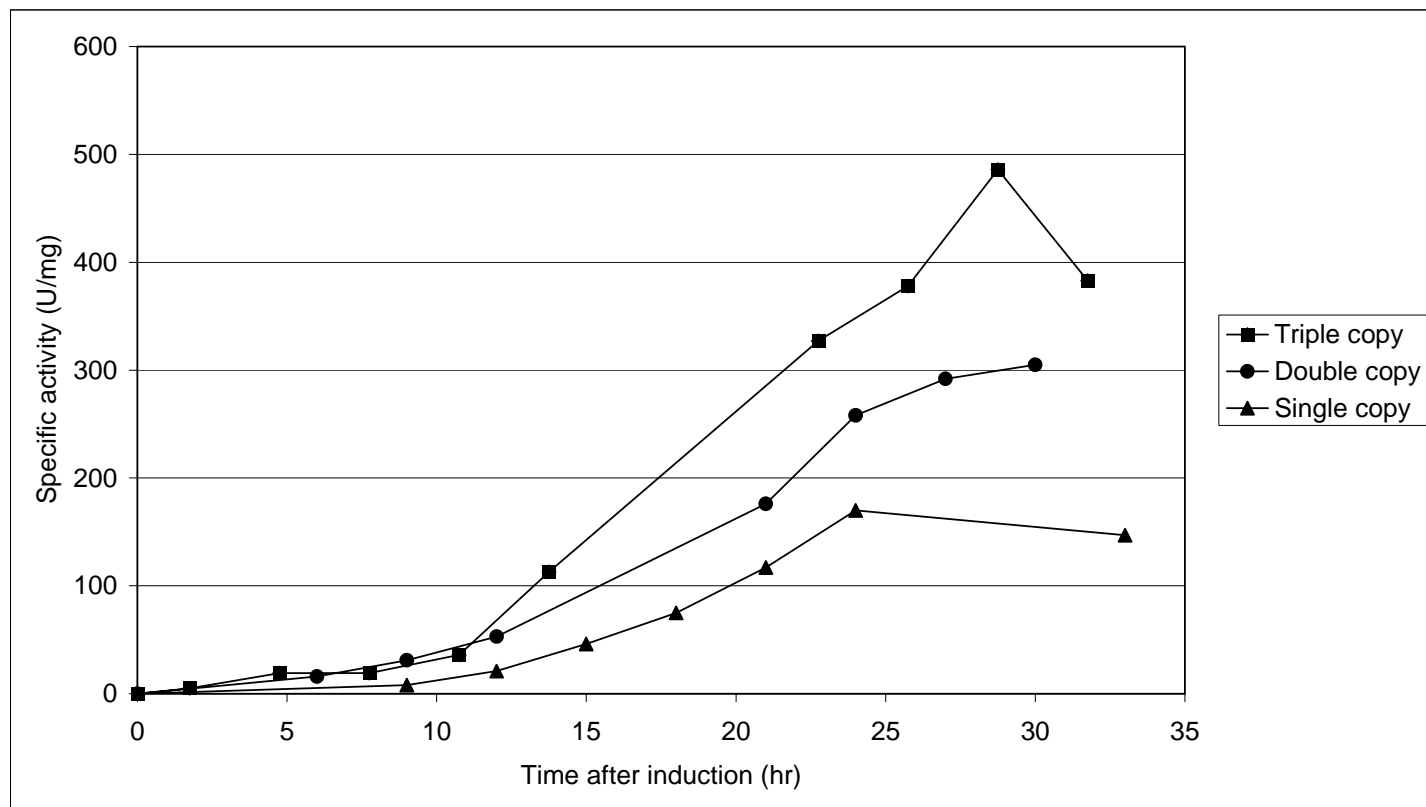
B



Introducing multiple copies of the $P_{phaP}::oph$ translational fusion increased the OPH expression levels in high cell density fermentations. Moreover, a proportional increase in OPH expression level was observed with increasing copy number.

Results from the fermentation are summarized in Fig 6. By integrating three copies of the $P_{phaP}::oph$ translational fusion into the chromosome, a titer of approximately 4.3 g/L was measured in high cell density fermentation. This represents the highest titer reported to date for this enzyme and is approximately 30 times greater than previously reported expression level in *E. coli* fermentations.

Fig 6: Summary of post-induction profiles for OPH expression in fermentations conducted with strain containing single, double and triple copies of the $P_{phaP}::oph$ translational fusion.



The results summarized in Section 5 culminated in a recent Biotechnology and Bioengineering publication (Srinivasan et al., 2003).

Scientific personnel:

A full time graduate student (Sriram Srinivasan) was supported by the grant.

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A Novel High-Cell-Density Protein Expression System Based on *Ralstonia eutropha*

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We describe the development of a novel protein expression system based on the industrial fermentation organism *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*) NCIMB 40124. This new system overcomes some of the shortcomings of traditional *Escherichia coli*-based protein expression systems, particularly the propensity of such systems to form inclusion bodies during high-level expression. Using a proteomics approach, we identified promoters that can be induced by simple process parameters or medium compositions in high-density cell culture or shake flasks, respectively. By combining newly developed molecular biological tools with a high-cell-density fermentation process, we were able to produce high levels (>1 g/liter) of soluble, active organophosphohydrolase, a model enzyme prone to inclusion body formation in *E. coli*.

Advances in protein engineering, the completion of numerous bacterial and fungal genome sequencing projects, and the isolation of new genes from extremophiles have led to an increased number of useful proteins. However, to enable recombinant proteins to play a role in applications where larger quantities are required, such as tissue engineering or catalytic materials, production technologies that are more efficient and robust are desirable. Improving protein production is the primary goal of recombinant microbial process development and is a focus of our laboratory. Overall protein productivity can be improved by increasing the product of two variables: (i) the amount of recombinant protein per cell (specific productivity) and (ii) the amount of cell mass per unit of volume and time (cell productivity). In order to improve volumetric productivity in a cost-effective manner, recombinant proteins are often produced in high-cell-density fermentations. High-cell-density fermentations offer many advantages over traditional fermentations in that final product concentrations are higher and downtime and water usage are reduced, yet overall productivity is improved, resulting in lower setup and operating costs (3, 6).

In the absence of specific folding or posttranslational modification requirements, *Escherichia coli* is usually the expression host of choice. *E. coli*-based fermentation systems produce good yields at laboratory scale; however, scale up to industrial scale, where oxygen enrichment, dialysis, and sophisticated feeding algorithms become impractical and cost prohibitive, has been challenging (22). Unlike laboratory-scale fermentors, where the above-mentioned techniques are feasible, large-scale industrial fermentors are limited by mixing constraints and their ability to transfer oxygen and heat. It is well documented that oxygen-limiting conditions in *E. coli* result in the production of reduced carbon metabolites such as acetate, lactate, and formate (1, 20), which accumulate in high-cell-density cultures and ultimately inhibit further microbial growth. Titers of recombinant proteins in *E. coli* are limited by

several factors including the final cell density (22), the tendency for inclusion bodies to be formed when strong promoters are used (15, 23), and proteolysis, which adversely affects the quality of the final product (14, 17).

Considerable efforts have been undertaken to overcome the limitations of *E. coli* as a recombinant-protein expression host, and improving the organism's fermentation performance has been the focus of many biochemical engineering research groups. The accumulation of organic acids during the fermentation process has been reduced by several approaches: (i) installation of inline dialysis membranes (12, 26), (ii) implementation of a controlled feeding regimen imposing a reduced growth rate on the microorganism (28), (iii) enrichment with pure oxygen (7), and (iv) engineering of a heme cofactor into the host organism (11). Inclusion body formation has been reduced by expression of a target protein in frame with a highly soluble protein, thereby creating a more soluble fusion protein, and/or by decreasing the growth rate of the organism by decreasing the temperature of the fermentation (39). Proteolytic activity has been reduced by the generation of protease-deficient mutants (18).

While considerable progress has been made in addressing some of these shortcomings, the performance of *E. coli* as a recombinant-protein expression host still leaves much to be desired. In this paper, we report the development of a novel prokaryotic protein expression system based on a nonpathogenic organism, *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*). The described system permits high-cell-density culture in a defined minimal medium, does not require the addition of antibiotics to maintain plasmid stability, and does not require exogenous addition of inducers such as isopropyl-B-D-thiogalactopyranoside (IPTG) to initiate protein expression. A model protein that has traditionally been difficult to obtain in soluble form in *E. coli* (organophosphohydrolase [OPH] from *Pseudomonas diminuta* MG) was produced at high yield without any measurable formation of inclusion bodies.

R. eutropha has been used at a scale of several hundred thousand liters for the production of polyhydroxyalkanoate (PHA), a biodegradable polymer, by ICI/Zeneca and later Monsanto. The genome has been sequenced (<http://jgi.doe>

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>R. eutropha</i> strains		
NCIMB 40124	Wild type, gentamicin resistant	NCIMB
GS5	NCIMB 40124 containing plasmid pKTTPPCm	This study
SS14	PHAP::OPH, <i>phaPp-OPH</i> translational fusion strain derived from NCIMB 40124 and pJQPPCm	This study
<i>E. coli</i> strains		
TOP10	Strain for ligation and cloning of genes	Invitrogen
S-17	Host strain for pJQ plasmids	10, 27
Plasmids		
pCR2.1-TOPO	High-copy-number plasmid for cloning; confers ampicillin and kanamycin resistance	Invitrogen
pJQ200mp18	Homologous recombination plasmid conferring gentamicin resistance	27
pJQPPCm	<i>phaPp-OPH</i> translational fusion cloned into pJQ200mp18	This study
pKNOCK-Cm	Plasmid for recombination, conferring chloramphenicol resistance	2
pKT230	Broad-host-range plasmid	American Type Culture Collection
pUC19	High-copy-number plasmid used for cloning; confers ampicillin resistance	New England Biolabs
pUCPPCm	<i>phaPp-OPH</i> translational fusion cloned into pUC19	This study

.gov/JGI_microbial/html/ralstonia/ralston_homepage.html), and numerous high-cell-density processes have been reported previously (13, 30). Unlike *E. coli* and other enterobacteria which preferentially metabolize hexose sugars through the Embden-Meyerhof-Parnas pathway, *R. eutropha* preferentially uses the Entner-Doudoroff pathway to metabolize hexoses (21, 34). This results in a higher NADPH/NADH ratio, which causes a higher degree of biosynthetic as opposed to respiratory reducing equivalents. This, we believe, positively affects protein formation, since *R. eutropha* has the ability to balance the reducing equivalents generated from carbon metabolism by using the production of polyhydroxybutyrate (PHB) as a sink for reducing power (i.e., NADPH) in the absence of a final electron acceptor such as oxygen. Both *E. coli* and *Bacillus* spp. are forced to synthesize reduced organic acids to balance their redox state under oxygen-limiting conditions, and these acid metabolites have been shown to negatively affect overall fermentation performance. In contrast, PHB is a high-molecular-weight polymer that causes no osmotic stress on the cell and does not adversely affect overall bacterial growth. High-cell-density fermentations of *R. eutropha* (up to 230 g/liter) in large-scale reactors have been described previously by various groups (31, 37). Most of this work has been aimed at maximizing the PHB content; hence, the real cell mass (real cell mass = total biomass – PHB) was generally below 30 to 40 g/liter.

OPH, the model enzyme. OPHs (EC 3.1.8.1) are enzymes that are able to hydrolyze and thereby substantially reduce the toxicity of cholinesterase inhibitors like sarin and VX. OPH is a 72-kDa homodimeric enzyme that requires a divalent cation such as cobalt or zinc (8). The enzyme catalyzes the hydrolysis of the organophosphorus compounds at a remarkable rate; hence, it has found applications in detoxification and decontamination of parathion-containing agricultural fields and chemical weapons stock (41). Other applications include its use as a biosensor for monitoring levels of these compounds and for protection against chemical warfare agents (29). Several attempts to express gram quantities of OPHs in *E. coli* and other organisms have been hampered by the formation of inclusion bodies and the inability to grow to high cell densities. Attempts to improve production by engineering a fusion pro-

tein with highly soluble proteins like green fluorescent protein have resulted in reduced specific productivity and require post-translational cleavage of the fusion (40, 41). To date, there has been no report of a fermentation process that provides sufficient amounts of OPH for large-scale applications (9). OPH was chosen for this study because it has a well-documented history of forming inclusion bodies in *E. coli* and thus seemed to be a suitable model protein (32, 33).

MATERIALS AND METHODS

Strains, plasmids, and oligonucleotides. The various strains and plasmids that were used in this study are listed in Table 1. The oligonucleotides used in this study were PromUp (dCAGGAATTCATCGCGCAGCATGC), PromDn (dGATGAGCTCTCTCCAGTGGTGAAC), ParUp (dCTCGAGCTCATGTCATCGGTACC), and ParDn (dGGAAGGATCCAGATGGCGTCAT) (the restriction sites engineered in the oligonucleotides are underlined).

Growth media and antibiotics. *E. coli* strains were grown in Luria-Bertani medium. *R. eutropha* strains were cultivated in one of the following media depending on the application: NR medium (36), PCT medium (Table 2), and Lee medium (25). NR is a complex medium, while both PCT and Lee media are

TABLE 2. Composition of PCT medium^a

Component	Concn (g/liter)
Glucose.....	500
Salts ^b	
MgSO ₄ · 7H ₂ O.....	2.2
K ₂ SO ₄	3.0
Na ₂ SO ₄	0.18
FeSO ₄ · 7H ₂ O.....	0.18
H ₃ PO ₄ (1.48 M).....	9.6 ^c
Trace elements	
CuSO ₄ · 5H ₂ O.....	0.2
ZnSO ₄ · 6H ₂ O.....	1.0
MnSO ₄ · 4H ₂ O.....	1.0
CaCl ₂ · 2H ₂ O.....	26.0

^a Glucose, salts, and trace elements were autoclaved separately, and 40 ml of glucose, 1 liter of salts, and 2.4 ml of trace elements were mixed at room temperature.

^b The pH was adjusted to 6.8 prior to autoclaving with 28% NH₄OH.

^c Milliliters per liter.

minimal media. Antibiotics were added to the growth media in the following concentrations: for *R. eutropha*, 10 μg of gentamicin and 50 μg of chloramphenicol/ml; for *E. coli*, 100 μg of ampicillin, 10 μg of gentamicin, 25 μg of kanamycin, and 50 μg of chloramphenicol/ml (all antibiotics were obtained from Sigma, St. Louis, Mo.).

Fermentation procedure. Fed-batch fermentations of *R. eutropha* NCIMB 40124 were carried out in a 3-liter fermentor (Applikon, Foster City, Calif.) under the following conditions: 30°C, 1.5 liter of airflow/min, and a 630-rpm stirrer speed with an initial working volume of 1 liter (1 \times PCT medium with 5% [vol/vol] inoculum). An Applikon programmable logic controller (ADI1030) was used for maintaining temperature at 30°C and pH at 6.8. The pH of the medium was maintained with 85% phosphoric acid and aqueous ammonia (28%). The concentration of dissolved oxygen was maintained at 30% by controlling the stirrer speed up to a maximum speed of 1,250 rpm, at which point the dissolved-oxygen concentration dropped below 30% and became a function of the glucose feed rate. The fermentation was carried out in a batch mode until the initial glucose (20 g/liter) was consumed. Following the batch phase, a feed containing glucose, phosphoric acid, $\text{CoCl}_2 \cdot 7\text{H}_2\text{O}$, and MgSO_4 (600 g/liter, 220 mM, 0.15 mM, and 19 mM, respectively) was used to promote linear growth until a biomass concentration of approximately 100 g/liter was achieved. The glucose feed rate was initially maintained at 11.7 g/h and after 18 h of feeding was increased to 14 g/h. Induction was initiated by changing the feed solution to glucose (600 g/liter) containing 15 mM CoCl_2 . The second glucose feed solution contained no phosphate, leading to a gradual depletion in the medium, which in turn induced the expression of OPH. Offgas was analyzed in real time for O_2 and CO_2 concentrations with a model 3750 carbon dioxide and oxygen analyzer (Illinois Instruments, Ingleside, Ill.).

HPLC analysis. Samples of supernatant from the fermentation were collected and analyzed by high-pressure liquid chromatography (HPLC) on an Aminex 87H column (Bio-Rad Laboratories, Hercules, Calif.) to determine the concentrations of phosphate, sulfate, glucose, acetate, lactate, and ethanol. Five millimolar sulfuric acid at a flow rate of 0.7 ml/min was used as the mobile phase. All six compounds were detected by measurement of the refractive index on a Waters 410 refractometer.

DNA preparation, manipulation, and Southern blot hybridization. Preparation and manipulation of DNA, genomic DNA preparation, transformation, and hybridization were carried out using standard procedures or by following manufacturers' instructions. Sequencing was performed at the Molecular Biology Core Facility at Dartmouth College.

Construction of integration plasmid pJQ200mp18Cm. pKNOCK-Cm (2) vector was digested with the restriction enzyme *Mlu*I and was separated by agarose gel electrophoresis. A 1.1-kb DNA fragment was purified from the gel, and the ends were blunted using T4 DNA polymerase (New England Biolabs, Beverly, Mass.). The fragment was inserted into the *Eco*RV site within the gentamicin resistance gene of plasmid pJQ200mp18. This plasmid was used for chromosomal integration into *R. eutropha* by homologous recombination.

Construction of the OPH expression cassette. A 450-bp sequence of *R. eutropha* DNA containing the promoter along with the ribosomal binding site of the *phaP* gene (GA24) was amplified using PCR with oligonucleotides PromUp and PromDn and was inserted into pUC19 as an *Eco*RI/*Sac*I insert. An 1,100-bp sequence corresponding to the coding region for OPH was amplified from pCMS75 with oligonucleotides ParUp and ParDn such that the ATG start site of the OPH gene was in the same position as that of *phaP* in the wild type. Plasmid pKNOCK-Cm was digested with the restriction enzyme *Mlu*I and separated by gel electrophoresis. A 1.1-kb DNA fragment was purified from the gel, and the ends were blunted using T4 DNA polymerase. The fragment was inserted into the *Hinc*II site of the pUC19 vector containing *phaPp*-OPH (*phaP* promoter carrying the coding region for OPH). The sequence of the construct was verified, and the plasmid was designated pUCPPCm.

Construction of OPH-producing *R. eutropha*. The 2.4-kb *Eco*RI/*Pst*I fragment containing *phaPp*-OPH-CAT (*phaP* promoter carrying the coding region for OPH together with the chloramphenicol acetyltransferase gene) of pUCPPCm was inserted into the broad-host-range plasmid pKT230, generating pKTPPCm. Similarly, the 2.4-kb *Fsp*I/*Pst*I fragment was inserted into the *Eco*ICRI/*Pst*I-digested plasmid pJQ200mp18, generating pJQPPCm. Gene transformation from *E. coli* into *R. eutropha* was accomplished by adaptation of standard biparental-mating protocols (27). Colonies that grew on minimal medium and were resistant to gentamicin and chloramphenicol were selected and subjected to a qualitative test for OPH activity. A small amount of cells was scraped from the plates and was resuspended in a 10 mM Tris-HCl buffer containing 133 μM parathion. The appearance of a yellow color was used as an indicator of the presence of OPH.

Enzyme activity assays. Cell pellets were resuspended in 150 mM CHES buffer [2-(cyclohexylamino)ethanesulfonic acid], pH 9.0, to an optical density at 600 nm (OD_{600}) between 1 and 3. The samples were sonicated in a sonic dismembrator 550 (Fisher Scientific, Fair Lawn, N.J.) in two pulsed cycles (2 s on, 0.5 s off, 30-s duration, 5 min of cooling on ice between cycles). One milliliter of this cell extract was centrifuged at $16,000 \times g$ for 5 min, and the supernatant was analyzed for enzyme activity against paraoxon (Sigma) as described previously (8, 32). An extinction coefficient of $17,000 \text{ M}^{-1}\text{cm}^{-1}$ for *p*-nitrophenol at pH 9.0 was used to calculate the activity. The total protein concentration was measured using a Bradford protein assay kit (Bio-Rad).

Western blots. Whole-cell pellets were resuspended in water to a final concentration of 0.2 g (dry cell weight)/liter. The cell suspension (20 μl) was added to 20 μl of 2 \times sodium dodecyl sulfate (SDS) cracking buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 0.2% bromophenol blue, 20% glycerol) and boiled for 10 min. Samples (15 μl) were then loaded on a Tris-HCl-SDS-12% polyacrylamide gel (Bio-Rad) and resolved at 100 V. The proteins were then transferred from the gel to a nitrocellulose membrane (0.2 μm ; Schleicher & Schuell, Keene, N.H.) for 1 h at 100 V/350 mA. The membrane was then blocked in a TBST solution buffer (50 mM Tris base, 188 mM sodium chloride, 0.05% Tween 20, pH 7.5) containing 3% bovine serum albumin (Sigma) and incubated for 2 h at room temperature. The membrane was then exposed to a crude primary rabbit anti-OPH serum (generously supplied by Janet Grimsley, Texas A&M University) diluted in the TBST buffer. After being washed repeatedly with TBST buffer, the membrane was exposed to a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Pierce, Rockford, Ill.) diluted in TBST buffer. After repeated washing with TBST buffer, color development was achieved in a Tris-buffered saline buffer containing 500 mg of DAB (di-amino benzinidine; Pierce)/liter and 100 μl of a 30% H_2O_2 solution (Fisher Scientific)/liter.

Quantification of PHB in *R. eutropha* cells. PHB was quantified by the sulfuric acid-D2O method of Karr et al. (19) with modifications (42).

2D SDS-PAGE and protein sequencing. Samples from the fermentation were collected and centrifuged. Cell pellets were dissolved in osmotic lysis buffer (10 mM Tris [pH 7.4], 0.3% SDS) containing nuclease stock and protein inhibitors. The protein stock (10 \times stock) contained 50 mM MgCl_2 , 100 mM Tris (pH 7.0), 500 μg of RNase (RNase A from bovine pancreas type IIIA; Sigma)/ml, and 1,000 μg of DNase (DNase I, type II from bovine pancreas; Sigma)/ml. Protease inhibitors (100 \times stock) contained 20 mM AEBSF (4-[2-aminoethyl] benzene-sulfonyl fluoride hydrochloride; Calbiochem, San Diego, Calif.), 1 mg of leupeptin (Sigma)/ml, 0.36 mg of E-64 (*N*-[*N*-(*L*-trans-carboxyoxiran-2-carbonyl)-*L*-leucyl]-agmatine; Sigma)/ml, EDTA (Calbiochem), and 5.6 mg of benzamide (Sigma)/ml. One milliliter of each sample was lyophilized. Resulting residues were redissolved to a concentration of 5 mg/ml in a 1:1 dilution of SDS boiling buffer (5% SDS, 5% β -mercaptoethanol, 10% glycerol, 60 mM Tris [pH 6.8]) and heated in a boiling-water bath for 2 min. Two-dimensional (2D) SDS-polyacrylamide gel electrophoresis (PAGE) was performed by Kendrick Labs, Inc. (Madison, Wis.) according to a method modified from that of O'Farrell. Isoelectric focusing was carried out in glass tubes with an inner diameter of 2.0 mm by using 2.0% BDH ampholines (pH 4 to 8; Hoefer Scientific Instruments, San Francisco, Calif.) at 9,600 V \cdot h. One microgram of an isoelectric focusing internal standard, tropomyosin protein, with a molecular weight (MW) of 33,000 and pI 5.2, was added to the samples. After equilibration for 10 min in buffer (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, 0.0625 M Tris [pH 6.8]), the tube gel was sealed to the top of a stacking gel on top of a 10% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was then carried out for 4 h at 12.5 mA. The following proteins (Sigma) were added as MW standards to the agarose that was used to seal the tube gel to the slab gel: myosin (MW, 220,000), phosphorylase A (MW, 94,000), catalase (MW, 60,000), actin (MW, 43,000), carbonic anhydrase (MW, 29,000), and lysozyme (MW, 14,000). These standards appeared as horizontal lines on the silver-stained 10% acrylamide slab gel. After slab gel electrophoresis, the gel was transferred to the transfer buffer and transblotted onto polyvinylidene difluoride paper overnight at 200 mA and approximately 100 V per two gels. The blots were stained with Coomassie brilliant blue R250 in 50% methanol and destained in 50% methanol. Analysis of 2D gels for differentially expressed polypeptides was conducted by eye. The N terminus (10 amino acids) of the identified protein spots was sequenced by Edman degradation at the Protein Chemistry Core Facility, Howard Hughes Medical Institute, Columbia University.

RESULTS

High-cell-density fermentation. We developed a simple, efficient, and reproducible high-cell-density fermentation proto-

col based on *R. eutropha* strain NCIMB 40124 that is aimed at maximizing the overall protein content in the reactor. We can routinely obtain cell densities of over 150 g (dry cell weight)/liter in a simple fed-batch mode (data not shown) in a minimal medium containing only inorganic salts and glucose. Previous studies have established that *R. eutropha* produces PHB under phosphate- or nitrogen-limited conditions when supplied with an appropriate carbon source such as glucose or fructose. We conducted a series of fermentation experiments to establish the amount of phosphate required to maximize cell mass formation while minimizing polymer accumulation. We found that by using various carbon/phosphate ratios in the feed, we were able to effectively control the PHB content of the cell in high-cell-density culture (data not shown). By using this feeding strategy, we were able to achieve a cell density of 182 g (dry cell weight)/liter, with low polymer content. *R. eutropha* has been shown to have a comparatively high protein content of 68% of total dry cell weight (16). Oxygen uptake rates were typically below $150 \text{ mmol} \cdot \text{liter}^{-1} \cdot \text{h}^{-1}$, well within reach of commercial-scale fermentors (5). To determine the extent of overflow metabolism under these conditions, we performed HPLC analysis of the fermentation broths at various times during the fermentation process (data not shown). Throughout the process, acetate, lactate, and ethanol were below the detection limit of 0.1 g/liter (data not shown). It should be noted that glucose feed rates were held constant over extended periods of the process, which greatly simplified process control and improved the reproducibility of the process. With biomass increasing in a roughly linear fashion, the specific growth rates were therefore gradually reduced from $\mu = 0.25 \text{ h}^{-1}$ to about $\mu = 0.025 \text{ h}^{-1}$.

Identifying strong inducible promoters. High-level expression of recombinant proteins requires the availability of a strong and preferably inducible promoter. On the large, commercial scale, the use of antibiotics (to ensure plasmid maintenance) and inducers such as IPTG (to induce expression) is not desirable for environmental and economic reasons. Thus, we sought to systematically identify genes that are up-regulated in *R. eutropha* during the fermentation process itself or are up-regulated in response to the variation of a simple process parameter. The induction events tested were as follows: (i) a change in temperature, (ii) the omission of phosphate in the medium feed, (iii) microaerobic conditions (oxygen limitation), (iv) change in pH, and (v) change in carbon source (glucose to fructose). In order to assess the change in the proteome in response to these induction events, we conducted several fermentations. A control fermentation without any induction event was used as the base case. In each of the fermentations, we grew the cells to an OD_{600} of approximately 300 and then altered one of the above-mentioned parameters and collected cell samples after imposing the specific changes. In three fermentation runs (pH change, temperature change, and oxygen limitation), high cell densities were not obtainable and thus were not considered further. In two of the five fermentation runs (phosphate limitation and change of glucose to fructose), we were able to achieve cell densities in excess of 150 g (dry cell weight)/liter. Cell samples from these fermentations were collected, and total cell protein was analyzed by 2D SDS-PAGE. By superimposing 2D SDS-PAGE gels containing protein samples obtained pre- and postinduction, we

were able to identify two proteins (glucose-to-fructose change [Fig. 1C] and phosphate limitation [Fig. 1D]) that were up-regulated in response to the imposed conditions and one protein that appeared to be expressed at high constitutive levels under all conditions (Fig. 1). Table 3 describes the three proteins denoted in Fig. 1. One protein that was significantly induced by phosphate limitation was found to be GA24, a product of the *phaP* gene, a phasin previously described by Wieczorek and coworkers (38). GA24 is a protein found on the surface of PHA granules that regulates the size of the granule. Wieczorek et al. also found that GA24 was expressed at relatively high levels (5% of the total protein) in *R. eutropha* during PHB production under nitrogen-limiting conditions. Since *phaP* was a promoter, inducible by a simple process parameter (phosphate limitation) and with low-level basal activity, it met our criteria for a suitable recombinant-protein expression promoter for high-cell-density culture. In order to determine whether quorum sensing or high cell density in itself affects a change in the proteome samples, we collected samples at OD_{600} values of 100 and 600 without changing any process parameters and then analyzed them by 2D SDS-PAGE (Fig. 1A and B).

OPH production in *R. eutropha*. Plasmid pJQPPCm, containing the open reading frame of the OPH gene under the control of *phaP*, was constructed and transformed into *E. coli* by electroporation. This plasmid was integrated into the *phaP* locus of *R. eutropha* by biparental mating, thereby providing a single stable chromosomal copy of the OPH gene. Three individual colonies were selected and analyzed for OPH production. PCR was performed with one internal and one external primer to verify the chromosomal location of integration. All three strains yielded PCR products consistent with a single chromosomal insertion (data not shown). In addition, Southern blotting was performed on one of the strains to confirm that only a single copy of the OPH open reading frame was received and that integration indeed occurred in the *phaP* locus (Fig. 2).

High-cell-density production of OPH. Although high OPH-specific activities have been obtained in *E. coli* in shake flask experiments, successful scale-up has failed due to inclusion body formation (33). In order to demonstrate that high levels of the enzyme can be produced in a simple and efficient fermentation process, we conducted a fermentation to produce approximately 100 g of biomass/liter and over 1 g of OPH/liter. The fermentation was conducted as a three-stage process: batch, fed batch, and induction. During the batch phase, the initial inoculum was allowed to grow such that the cells consumed the initial 20 g of glucose/liter. Glucose depletion (14 h after inoculation) was indicated by a sharp increase in the concentration of dissolved oxygen. After depletion of the initial glucose, a feed containing glucose, phosphate, and sulfate at a constant feed rate of 11.7 g of glucose/h was initiated. The cells continued to grow exponentially until a fixed growth rate corresponding to the glucose feed rate was achieved. The feed rate was then further increased to 14.0 g of glucose/h, thereby increasing the growth rate. During this phase, both phosphate and sulfate concentrations increased in the fermentation, whereas glucose as the rate-limiting nutrient remained undetectable due to its immediate consumption in the reactor. During this phase, the amount of PHB in the cell remained less

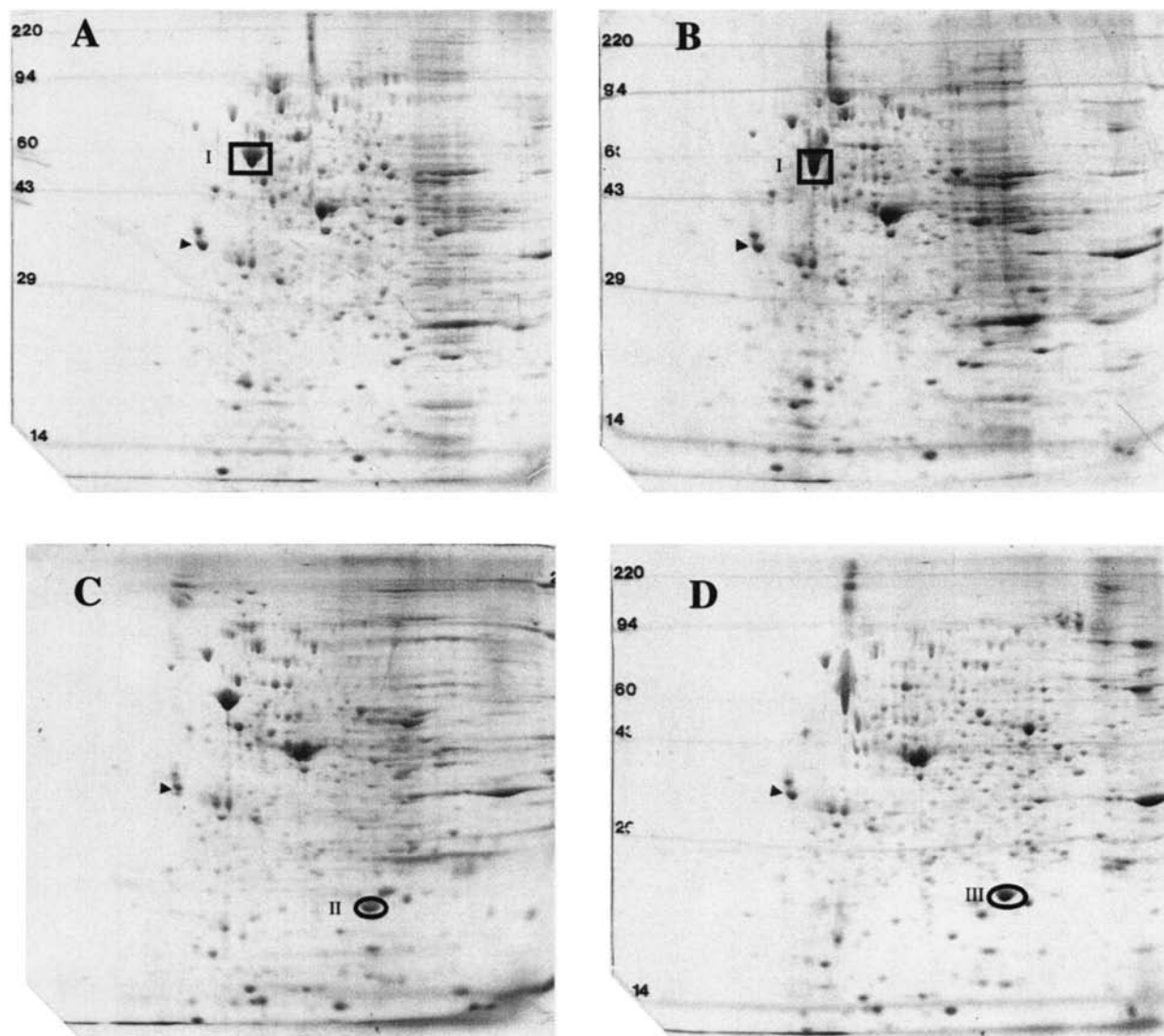


FIG. 1. 2D gel electrophoresis of whole-cell extracts of *R. eutropha* from three separate fermentation experiments: fed-batch fermentation on glucose minimal medium. Samples were collected at OD₆₀₀ values of 100 (A) and 600 (B), after changing the carbon source from glucose to fructose at an OD₆₀₀ of 300 and sampling at an OD₆₀₀ of 450 (C), and after changing the carbon source to a phosphate-free glucose feed at an OD₆₀₀ of 300 and sampling at an OD₆₀₀ of 450 (D). Samples shown in panels A and B were collected from the same fermentation experiment at different times; samples shown in panels C and D were collected from separate fermentation experiments. Cell pellets from the fermentation were collected and lysed. The samples were resuspended in SDS buffer, and 2D SDS-PAGE was run with each individual sample. The gels were visually analyzed, and three proteins of interest were identified: a constitutively expressed protein (I), a fructose-induced protein (II), and a protein induced by phosphate limitation (III). Molecular masses (in kilodaltons) are indicated. The small arrowheads denote an internal standard, the protein tropomyosin (MW, 33,000; pI 5.2).

than 5% of the total biomass. Despite some PHB production, the basal activity of the *phaP* promoter was low, as determined by enzyme assays (Fig. 3B) and Western blotting (Fig. 4). A biomass concentration of 93.4 g/liter was obtained 38 h after

inoculation (24 h after initiating the glucose feed). At this point, the feed was changed to a glucose solution, containing 15 mM cobalt but lacking phosphate, to initiate the induction phase. OPH requires a divalent cation for enzymatic activity

TABLE 3. N-terminal sequence and protein similarity

Protein	N-terminal sequence	Protein similarity	Function
I	AAKDVFVGDA	GroEL (<i>Burkholderia cepacia</i>)	Chaperon
II	TOXTAEQCTK	No similarity identified	
III	VILTPEQVAA	GA24 (<i>R. eutropha</i>) (also known as PhaP protein)	Surface protein bound to PHB (an intracellular polymer) ^a

^a GA24 represents 5% of total protein (35, 38).

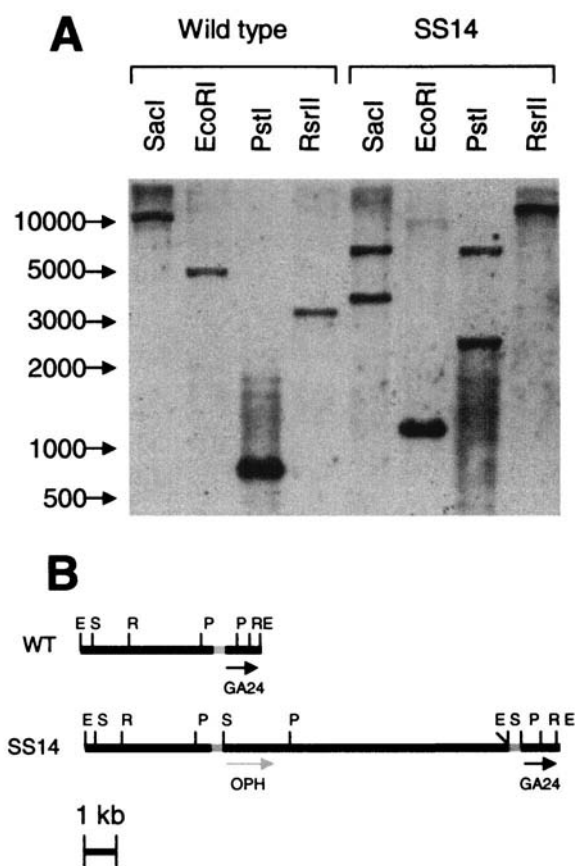


FIG. 2. Southern blot analysis of wild-type *R. eutropha* and *R. eutropha* SS14. Genomic DNA from the wild type and integrant SS14 were extracted and digested with *SacI*, *PstI*, *EcoRI*, and *RsrII*. (A) Results of Southern blot analysis of genomic DNA of the wild type and the integrant probed with a horseradish peroxidase-labeled *phaP* promoter. The sizes of the markers (base pairs) are indicated on the left. (B) Map of the analyzed genomic region in the wild type and the integrant, indicating the probe and the various restriction enzymes used. S, *SacI*; E, *EcoRI*; P, *PstI*; R, *RsrII*. The open reading frames of OPH and GA24 (*phaP*) are also indicated.

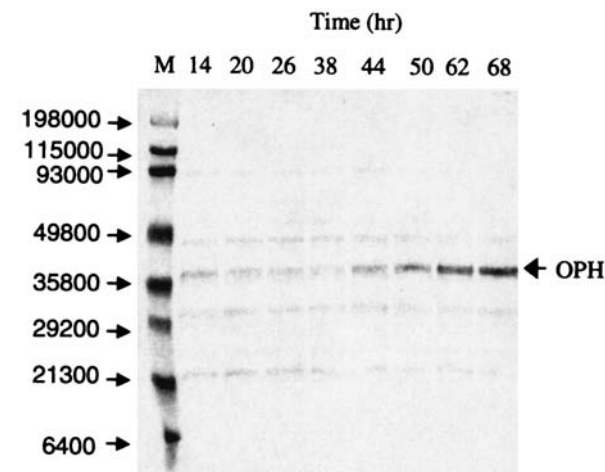
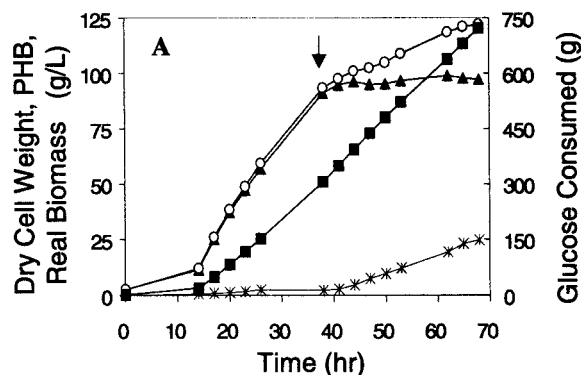


FIG. 4. Western blot analysis of fermentation samples. Total cell pellets were diluted to approximately 0.2 g (dry cell weight)/liter and then boiled on SDS-PAGE cracking buffer and resolved on a 12% polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane, and blotting was done with anti-OPH antibody. The figure shows the time course of OPH production. M, broad-range MW marker, with MWs indicated on the left.

(25), so cobalt was added to the glucose feed. During the initial induction phase, the phosphate concentration rapidly decreased in the reactor (Fig. 3B), and upon depletion, OPH production was induced. After 30 h of induction (68 h after inoculation), the specific activity of the protein was found to be 145 U/mg (Fig. 3B). In addition to the results of the enzyme activity assays, the induction of OPH could also be seen in the appearance of a band corresponding to the MW of OPH (approximately 36,000) in the Western blot (Fig. 4, lanes corresponding to 44 to 68 h). In this fermentation, we achieved a concentration of 99.1 g of real biomass/liter. The specific activity of pure OPH (with cobalt as the divalent cation) has been reported to be 7,250 U/mg (29) and 8,020 U/mg (24) using the paraoxon enzyme activity assay; thus, the specific activity of 145 U/mg corresponds to 2.0 or 1.8% of total cellular protein,

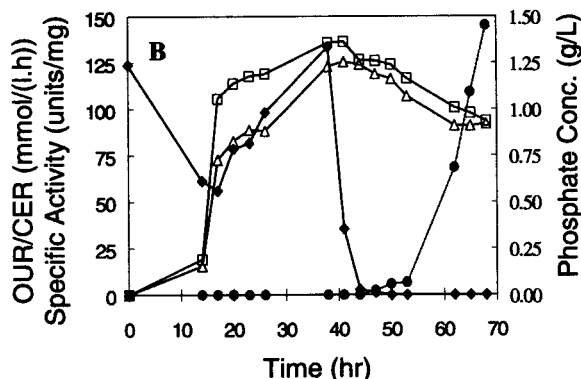


FIG. 3. OPH production in high-cell-density fermentation of *R. eutropha*. (A) The dry cell weight, real biomass, PHB, and glucose feeding profile for a typical high-cell-density fermentation. The arrow indicates the time of induction. Symbols: open circles, total dry cell weight; closed triangles, real biomass; crosses, PHB; closed squares, glucose consumed. (B) The oxygen uptake rate (OUR), the carbon dioxide evolution rate (CER), the phosphate concentration in the reactor, and the specific activity of OPH produced. Symbols: open squares, OUR; open triangles, CER; closed diamonds, phosphate concentration; closed circles, specific activity.

respectively. As previously noted, the protein content of *R. eutropha* has been found to be 68% of dry cell weight (15). The product of 99.1 g of biomass/liter, 0.68 g of protein/g of biomass, and 0.018 g of OPH/g of protein yields an OPH concentration of 1.2 g/liter.

DISCUSSION

Traditionally, the development of recombinant-protein production systems has focused on improving the fermentation processes of organisms for which molecular biological tools (e.g., transformation protocols, selection markers, inducible promoters, etc.) have already been established. In this study, we used an organism with robust fermentation characteristics and developed the molecular biological tools to produce recombinant proteins at high levels. We achieved cell densities of over 150 g/liter in a simple process that does not require complex medium components or the supplementation of air with pure oxygen. The process can be implemented manually or by a simple programmable logic controller. In the described fermentation process, growth-inhibiting organic acids were not synthesized although the levels of dissolved oxygen remained at 0% throughout most of the process.

Promoter traps have been used previously to identify strong promoters (4). However, the specific dynamic environment of a high-cell-density culture cannot be adequately reproduced on solid media. In particular, differentially expressed genes that respond to subtle environmental stimulants, or growth-phase-dependent genes, are not readily detected on solid media by use of promoter traps. Thus, we decided to isolate promoters that are up-regulated in the environment in which we expect to use these promoters, namely high-cell-density culture. By looking at the entire proteome before and after changing specific environmental conditions, we identified an inducible promoter in *R. eutropha* that could be regulated with a simple process parameter (i.e., phosphate limitation). Previous work using shake flasks has shown that *phaPp* is induced under PHB production conditions and that expression is controlled by a DNA binding protein, PhaR (43).

In this study, we were able to obtain 1.2 g of soluble active OPH/liter in a simple high-cell-density fermentation process. Although only a single chromosomal copy of the OPH gene was present, it represents the highest OPH titer reported to date (33). In a way very similar to the concept of man-hours, where the product of two variables determines overall productivity, *R. eutropha* fermentations allow more cells to produce protein at a lower specific rate while achieving higher overall productivities. This reduces the likelihood of inclusion body formation and thus allows for the higher recovery of soluble active protein. However, to further improve expression levels and to test the limits of soluble-protein production, we are currently investigating the influence of gene dosage and artificial promoters.

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Production of Recombinant Proteins Using Multiple-Copy Gene Integration in High-Cell-Density Fermentations of *Ralstonia eutropha*

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Abstract: We have previously reported the development of a novel protein expression system based on *Ralstonia eutropha*. In this study we report on the influence of gene copynumber on recombinant protein expression in *R. eutropha*. We compare recombinant gene stability and expression levels of chromosomal integration with a plasmid-based expression system. Single, double, and triple copies of a gene encoding organophosphohydrolase (OPH), an enzyme prone to inclusion-body formation in *E. coli*, were integrated into the *R. eutropha* chromosome. A linear increase between the concentration of soluble, active OPH and gene copynumber was found. Using a triple-copy integrant, we were able to produce approximately 4.3 g/L of OPH in a high-cell-density fermentation. This represents the highest titer reported to date for this enzyme, and is approximately 30 times greater than expression levels reported in *E. coli*. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* 00: 000–000, 2003.

Keywords: protein expression; gene dosage; high cell density; fermentation

INTRODUCTION

High-cell-density fermentations have been used extensively for increasing the productivity and titer of recombinant proteins (Chen et al., 1992; Cutayar and Poillon, 1989; Knorre et al., 1991; Panda et al., 1999). Protein production in fermentation processes depends on several factors, including promoter strength (Wilms et al., 2001), induction time (Wu et al., 2001), gene dosage, and stability of expression construct (Grabherr et al., 2002). Various studies have been conducted to maximize the recombinant protein yield for *Escherichia coli*-based fermentations (Chae et al., 2000; Grabherr et al., 2002; Portner and Markl, 1998). These have resulted in tremendous improvements in titer and in amount of soluble protein. However, high-cell-density fermentations of *E. coli* suffer from various draw-

backs, including the formation of organic acids (Akeson et al., 1999; Kleman and Strohl, 1994), proteolysis (Han et al., 1999), and inclusion-body formation (Han et al., 1999; Makrides, 1996).

To overcome the shortcomings of *E. coli* fermentations, a novel high-cell-density fermentation based on *Ralstonia eutropha* has been developed in our laboratory (Srinivasan et al., 2002). This system has been shown to produce high levels of soluble recombinant protein in a simple, scalable, high-cell-density fermentation. Using this system, >1 g/L of soluble recombinant organophosphohydrolase (OPH) can be produced by a single chromosomal copy of OPH under the control of an inducible phosphate-responsive promoter.

In prokaryotes, high-level protein expression has been achieved by modulating gene dosage through the use of medium- or high-copynumber plasmids. However, an increase in plasmid copynumber usually increases the metabolic burden on the cell. For example, a 200-copy, 5-kb plasmid represents approximately 1 Mb of DNA, constituting 20% of the bacterial genome. The effect is compounded in a high-cell-density culture and results in unstable and heterogeneous fermentation. This reduces the protein yield and results in inclusion-body formation and proteolysis (Lee, 1996). In lower eukaryotes, especially *Pichia pastoris*, high levels of protein expression have been achieved by multiple random integration of the gene of interest. Studies have shown that an increase in gene dosage results in an increase in recombinant protein production up to a copynumber of 15 (Werten et al., 1999).

To better understand the effect of gene dosage and its impact on fermentation characteristics, we constructed strains containing single, double, and triple copies of the expression construct integrated into the chromosome. We also constructed a strain containing a multicopy plasmid with the expression construct. In this study, we report the effect of multiple copies on protein expression and stability of the constructs in the genome.

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MATERIALS AND METHODS

Strains, Plasmids, and Media

All *E. coli* and *R. eutropha* strains and plasmids used in this study are described in Table I. All pKNOCK series of vectors were transformed in *E. coli* S-17, whereas other vectors were manipulated in *E. coli* TOP10. *E. coli* strains were grown in Luria–Bertani (LB) media. *R. eutropha* strains were cultivated in tryptic soy broth (TSB) media (Difco Laboratories, Detroit, MI) or PCT media (Srinivasan et al., 2002). Antibiotics were added to growth media to the following final concentrations: for *R. eutropha*, gentamicin (10 µg/mL) and chloramphenicol (50 µg/mL); and for *E. coli*, ampicillin (100 µg/mL), gentamicin (10 µg/mL), and chloramphenicol (50 µg/mL) all antibiotics were obtained from Sigma Co. (St. Louis, MO).

DNA Preparation, Manipulation, and Southern Blot Hybridization

Preparation (Miniprep Kit, Qiagen, Inc., Valencia, CA) and manipulation of DNA, genomic DNA preparation (Geno-

mic-tip 500, Qiagen), transformation, and hybridization (North2South direct HRP labeling and detection kit; Pierce, Rockford, IL) were carried out using standard procedures (Sambrook and Russell, 2001) or the manufacturer's instructions. Sequencing was performed at the Molecular Biology Core Facility at Dartmouth College. PCR products were cloned into pCR2.1-TOPO vector (Invitrogen Corp., Carlsbad, CA) and then cut with the appropriate restriction enzymes.

Construction of Plasmids

A list of primers used in the study is shown in Table I. A 100-bp transcription terminator was amplified by PCR from the vector pEZSeq (Lucigen Corp., Middleton, WI) using the primers TERMplus and TERMminus. The construct was then cloned into pCR2.1-TOPO yielding pTOPO-Terminator. This vector was then cut with the restriction enzymes *Bgl*III/*Pst*I and cloned into the *Bam*HI/*Pst*I fragment of vector pUCPPCm, generating pUCPPT (contains the PPT expression cassette: GA24 promoter-OPH open reading frame-transcriptional terminator).

Three separate PCRs were performed to amplify the

Table I. Strains, plasmids, and primers used in this study.

Strain or plasmid	Description	Reference or source
<i>R. eutropha</i> strains		
NCIMB 40124	Wild-type	NCIMB
GS5	<i>R. eutropha</i> strain containing plasmid pKTPPCm	Srinivasan et al. (2002)
SS17	Single-copy integrant of the OPH expression cassette derived from NCIMB40124 and pKNOCK-PPT ¹	This study
SS18	Double-copy integrant of the OPH expression cassette derived from NCIMB40124 and pKNOCK-PPT ²	This study
SS19	Triple-copy integrant of the OPH expression cassette derived from NCIMB40124 and pKNOCK-PPT ³	This study
<i>E. coli</i> strains		
TOP10	Strain for ligation/cloning of genes	Invitrogen
S-17	Host strain for pKNOCK series of plasmids	Delorenzo et al. (1990)
Plasmids		
pCR2.1-TOPO	High-copynumber plasmid for cloning PCR products	Invitrogen
pEZSeq	High-copynumber plasmid with strong transcription terminators	Lucigen
pKNOCK-Cm	Plasmid for recombination conferring chloramphenicol resistance	Alexeyev (1999)
pKNOCK-PPT ¹	pKNOCK-Cm plasmid containing one copy of the OPH expression cassette (PPT)	This study
pKNOCK-PPT ²	pKNOCK-Cm plasmid containing two copies of the OPH expression cassette (PPT)	This study
pKNOCK-PPT ³	pKNOCK-Cm plasmid containing three copies of the OPH expression cassette (PPT)	This study
pKTPPCm	<i>phaPp</i> -OPH translational fusion and <i>CAT</i> gene cloned into plasmid pKT230	Srinivasan et al. (2002)
pTOPO-PPTa	pCR2.1-TOPO plasmid containing PPT expression cassette with <i>Sac</i> II/ <i>Pst</i> I cloning sites	This study
pTOPO-PPTb	pCR2.1-TOPO plasmid containing PPT expression cassette with <i>Pst</i> I/ <i>Hind</i> III cloning sites	This study
pTOPO-PPTc	pCR2.1-TOPO plasmid containing PPT expression cassette with <i>Hind</i> III/ <i>Xho</i> I cloning sites	This study
pTOPO-PPT ²	pTOPO-PPTc plasmid containing PPT expression cassette from pTOPO-PPTb	This study
pTOPO-Terminator	pCR2.1-TOPO plasmid containing transcription terminator from pEZSeq	This study
pUC19	High copynumber plasmid used for cloning, confers ampicillin resistance	New England Biolabs
pUCPPCm	<i>phaPp</i> -OPH translational fusion and <i>CAT</i> gene cloned into pUC19	Srinivasan et al. (2002)
pUCPPT	pUC19 vector containing the <i>phaPp</i> -OPH-terminator (PPT) expression cassette	This study
Primers		
TERMplus	5'-GAGAGATCTTAATAAATTTAAATCATACCTGACCT-3'	
TERMminus	5'-GAGCTGCAGATTTCATTATGGTGAAAGTTGGAA-3'	
mulSPEplus	5'-GCCACTAGTATCGCGCAGCATGCTGTACTTGCGC-3'	
mulPSTminus	5'-GCCCTGCAGATTTCATTATGGTGAAAGTTGGAAC-3'	
mulPSTplus	5'-GCCCTGCAGATCGCGCAGCATGCTGTACTTGCGC-3'	
mulHINDminus	5'-GCCAAGCTTATTTCATTATGGTGAAAGTTGGAAC-3'	
mulHINDplus	5'-GCCAAGCTTATCGCGCAGCATGCTGTACTTGCGC-3'	
mulXHominus	5'-GCCCTCGAGATTTCATTATGGTGAAAGTTGGAAC-3'	

1600-bp product containing the expression cassette with the following pairs of primers: (a) mulSPEplus and mulPSTminus; (b) mulPSTplus and mulHINDminus; and (c) mulHINDplus and mulXHOminus, using pUCPPT as the template. The products were then cloned into pCR2.1-TOPO vector generating pTOPO-PPTa, pTOPO-PPTb, and pTOPO-PPTc. The insert from pTOPO-PPTc was cut with *HindIII* and cloned into the pTOPO-PPTb vector, digested with *HindIII*, and dephosphorylated with calf intestinal phosphatase (CIP), generating the pTOPO-PPT² vector. pTOPO-PPT² contains two copies of the expression cassette (PPT).

The pTOPO-PPTa vector was cut with *SacII/PstI* and the PPT fragment was cloned into the *SacII/PstI* fragment of vector pKNOCK-Cm, generating pKNOCK-PPT¹. Similarly, TOPO-PPT² was cut with *PstI/XhoI* and cloned into pKNOCK-Cm and pKNOCK-PPT¹ cut with the same enzymes, generating the vectors pKNOCK-PPT² and pKNOCK-PPT³. Thus, three vectors, pKNOCK-PPT¹, pKNOCK-PPT², and pKNOCK-PPT³, containing one, two, and three copies of the PPT expression cassette, respectively, were generated. These vectors were then transformed into *R. eutropha* by biparental mating as described by Srinivasan et al. (2002). Eight colonies from each transformation were selected and tested for the presence of OPH activity. For each copy number, one positive strain was selected, designated SS17, SS18, or SS19, containing one, two, or three copies of the expression cassette, respectively. (All restriction enzymes and modifying enzymes were obtained from New England Biolabs, Beverly, MA.)

Fermentation and Enzyme Assay

Fermentations with *R. eutropha* strains were performed according to a previously described method (Srinivasan et al., 2002). Fed-batch fermentations were carried out in a 3-L fermentor (Applikon, Foster City, CA) at 30°C, with a 1.5-L/min airflow, 630-rpm initial stirrer speed, and an initial working volume of 1 L (1× PCT media with 5% [v/v] inoculum). An Applikon programmable logic controller (ADI1030) was used for maintaining temperature at 30°C and pH 6.8. Media pH was maintained with aqueous ammonia (28%). Dissolved oxygen concentration was maintained at 30% by controlling the stirrer speed up to a maximum speed of 1250 rpm, at which point the dissolved oxygen concentration dropped below 30% and became a function of the glucose feed rate. Fermentation was carried out in a batch mode until the initial glucose (20 g/L) was consumed.

Following the batch phase, a feed containing glucose, phosphoric acid, CoCl₂ · 7H₂O, and MgSO₄ (600 g/L, 220 mM, 0.15 mM, and 19 mM, respectively) was used to promote linear growth until a biomass concentration of approximately 100 g/L dry cell weight was achieved. The glucose feed rate was initially maintained at 11.7 g/h and, after 18 h of feeding, was increased to 14.0 g/h. Induction was initiated by changing the feed solution to glucose (600

g/L) containing 15 mM CoCl₂. The second glucose feed solution contained no phosphate, which led to a gradual depletion in the medium, which in turn induced OPH expression. Off-gas was analyzed in real time for O₂ and CO₂ concentrations with a CO₂/O₂ analyzer (Model 3750, Illinois Instruments, Ingleside, IL).

Fermentation with the plasmid-bearing strain was performed with a supply of chloramphenicol (80 mg/L in the initial media and 400 mg/L in the feed) to maintain selection pressure during the fermentation. Plasmid stability was measured by plating dilutions of the samples on Luria–Bertani (LB) plates with and without chloramphenicol. The plates were incubated at 30°C for 2 days. The stability was measured as the ratio of the number of colonies in the plate containing chloramphenicol to that of the number of colonies in the antibiotic-free plate. Cell viability was also measured by the number of colonies in the antibiotic-free plate.

OPH enzyme assays were performed according to the method of Serdar and Gibson (1985), with modifications (Srinivasan et al., 2002).

Western Blots

Whole cell pellets were resuspended in water to a final concentration of 0.2 g (dry cell weight)/L. The cell suspension (20 µL) was added to 20 µL of 2× sodium dodecyl-sulfate (SDS) lysis buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 0.2% bromophenol blue, 20% glycerol), and boiled for 10 min. Samples (15 µL) were then loaded on a Tris-HCl SDS–12% polyacrylamide gel (Bio-Rad, Hercules, CA) and resolved at 100 V. The proteins were then transferred from the gel to a nitrocellulose membrane (0.2 µm, Schleicher–Schuell, Keene, NH) for 1 h at 100 V/350 mA. The membrane was then blocked in a TBST solution buffer (50 mM Tris base, 188 mM sodium chloride, 0.05% Tween-20 [pH 7.5]) containing 3% bovine serum albumin (BSA; Sigma Co.) and incubated for 2 h at room temperature. The membrane was then exposed to a crude primary rabbit anti-OPH serum (generously supplied by Dr. Janet Grimsley, Texas A&M University) diluted in the TBST buffer. After repeated washing with TBST buffer, the membrane was exposed to a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Pierce Co., Rockford, IL), diluted 1:5000 in TBST buffer. After repeated washing with TBST buffer, color development was achieved in a Tris-buffered saline (TBS) buffer containing 500 mg/L diaminobenzidine (DAB; Pierce) and 100 µL/L of a 30% H₂O₂ solution (Fisher, Fair Lawn, NJ).

Quantification of Polyhydroxybutyrate in *R. eutropha* Cells

Polyhydroxybutyrate (PHB) was quantified by the sulfuric acid–high-performance liquid chromatography (HPLC) method of Karr et al. (1983), with modifications (York et al., 2001).

RESULTS AND DISCUSSION

High-Cell-Density Fermentation With Strain GS5

A high-cell-density fed-batch fermentation was carried out with the *R. eutropha* strain GS5 containing the plasmid pKTPPCm. This plasmid is a derivative of pKT230, which has been shown to have approximately ten copies per cell in *R. eutropha* (Jackson and Srien, 1999). The stability of the plasmid, cell viability, and OPH expression levels were monitored during the course of the fermentation (data not shown). During the batch phase approximately 50% of the cells lost the plasmid and only 10% of the cells contained the plasmid at the end of the fermentation (as measured by resistance to chloramphenicol). Cell viability also dropped significantly during the induction phase. However, OPH expression levels increased in the induction phase and reached a maximum specific activity of approximately 240 U/mg. This represents a 50% increase of protein concentration relative to a single chromosomal copy of the OPH gene. We hypothesize that, although the majority of the cells had lost the plasmid, the subpopulation that maintained the plasmid compensated for the loss by expressing a significant amount of OPH. Hence, single-copy gene integration does not saturate the inherent capability of recombinant protein production in *R. eutropha*. To further analyze the extent of transcriptional limitation, we constructed strains containing multiple gene-expression constructs integrated into the chromosome.

Construction of Multiple-Copy Gene-Expression Strains

Multiple gene integrations for recombinant protein production have been used in yeast to increase protein expression levels. However, this approach has not been widely used in prokaryotes, mostly due to the availability of multiple-copynumber plasmids. We constructed single-, double-, and

triple-copy integrations of the OPH expression cassette in *R. eutropha* to evaluate protein expression levels. An expression cassette, PPT, was made that contained the open reading frame of OPH under the control of the GA24 promoter and a strong transcriptional terminator. Because the copies were integrated in tandem, the transcriptional terminator sequence was used to reduce the synthesis of polycistronic messages. The expression cassette was cloned into the integration vector pKNOCK-Cm, generating three separate vectors containing one, two, or three copies of the OPH gene. *R. eutropha* wild-type strain was then transformed with these vectors and colonies were selected on a minimal media plate containing chloramphenicol and gentamicin. One colony for each copynumber (SS17, SS18, and SS19) was then selected and Southern blots were performed to confirm the copynumber and region of integration (data not shown). These strains were then evaluated for the OPH expression in a high-cell-density fermentation.

High-Cell-Density Fermentation With Multiple-Copy Integrants

High-cell-density fermentations (Srinivasan et al., 2002) were performed with strains SS17, SS18, and SS19. A typical fermentation involves a batch phase, a growth phase, and an induction phase. During the batch phase, the initial supply of glucose (20 g/L) is consumed. This was followed by a fed-batch growth phase, during which a biomass concentration of approximately 100 g/L (dry cell weight) is achieved. The PHB concentration during this phase remains at <5% of the total biomass (Fig. 1). Induction of the OPH gene is accomplished by limiting the phosphate concentration in the feed. A typical fermentation (SS19, the strain containing three copies of PPT) is shown in Figure 1. In this fermentation, the final concentration in biomass was 130.6 g/L (107.3 g/L real biomass and 23.3 g/L PHB). This indicates that gene dosage does not negatively affect the fermentation capability of *R. eutropha*.

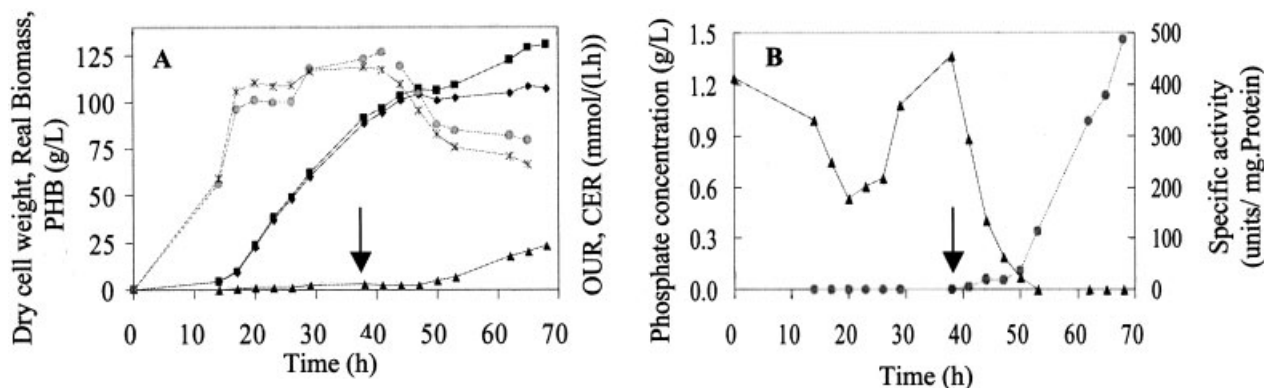


Figure 1. A typical high-cell-density fermentation of *R. eutropha*. (A) Dry cell weight, real biomass, PHB, O_2 uptake rate (OUR), and CO_2 evolution rate (CER) profile for high-cell-density fermentation with strain SS19 (*R. eutropha* strain with three copies of the expression construct). The arrow indicates the time of induction. Symbols: (■) total dry cell weight (g/L); (◆) real biomass (g/L); (▲) PHB (g/L); (○) oxygen uptake rate [OUR, mmol/(L · h)]; (X) carbon dioxide evolution rate [CER, mmol/(L · h)]. (B) Phosphate concentration (g/L) in the reactor and the specific activity of organophosphohydrolase produced (U/mg). Symbols: (▲) phosphate concentration; (○) specific activity.

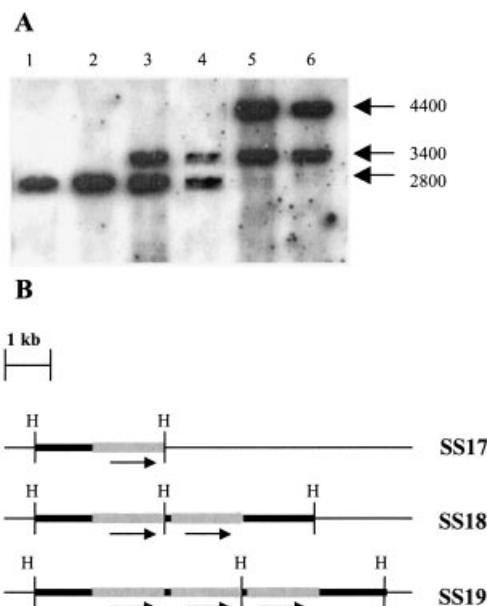


Figure 2. Southern blot analysis of *R. eutropha* strains SS17, SS18, and SS19. Genomic DNA from strains single-copy (SS17), double-copy (SS18), and triple-copy (SS19) integrants were extracted from samples collected in the initial phase (approximately 50 g/L biomass) and after induction (approximately 125 g/L biomass), and digested with *Hind*III. A 1-kb *OPH* open reading frame sequence (the *Sac*I/*Bam*HI fragment of plasmid pUCPPCm) was used to probe the blot. (A) Results of Southern blot analysis of genomic DNA of strains probed with a horseradish peroxidase-labeled *OPH* open reading frame. Lanes 1, 3, and 5 correspond to genomic DNA before induction and lanes 2, 4, and 6 correspond to genomic DNA after induction, for single-, double-, and triple-copy integrants. The sizes of the markers are indicated on the left. (B) Map of the genomic region analyzed in strains SS17, SS18, and SS19, indicating the expression construct (PPT) and the *Hind*III restriction enzyme sites. H, *Hind*III. The open reading frame of organophosphohydrolase (*OPH*) is also indicated.

Stability of Integration Constructs

Recombination of the integration locus during the course of the fermentation is of concern because of the large number of generations (approximately 25 generations). Moreover,

direct repeats of DNA loci are known to be genetically unstable. Recombination can be prevented by knocking out recombination genes like *recA*, *recE*, etc. (Amundsen et al., 2000; Hou and Hill, 2002). Because transformation in *R. eutropha* is achieved using homologous recombination, *recA*-deficient strains would not be suitable for cloning. To evaluate the recombination frequency between the multiple copies of *OPH*, a Southern blot was performed on samples collected during the initial growth phase and late induction phase (Fig. 2). The results show that multiple copies were stably integrated and recombination during the fermentation was absent. We suspect that multiple-copy integrations can be used successfully in other prokaryotic expression systems to achieve stable protein production in high-cell-density fermentation.

Comparison of *OPH* Expression

High-level expression of soluble *OPH* in *E. coli* has been unsuccessful due to the formation of inclusion bodies (Serdar et al., 1989; Wu et al., 2000). We investigated the effect of gene dosage to determine if soluble *OPH* expression can be saturated by multiple chromosomal integrants in *R. eutropha*. Figure 3A shows the specific activity of *OPH* produced during the induction phases of the three fermentations with single, double, and triple copies. In addition to enzyme activity assays, *OPH* expression levels can be observed as a 36-kDa band in a Western blot, with samples from before and after induction for the three fermentations (Fig. 3B). A linear increase in the amount of *OPH* with gene dosage was observed, whereas basal level expression remained low (maximum specific activities of *OPH* of 170, 312, and 487 U/mg for one-, two-, and three-copy integrations, respectively). After 30 h of induction (68 h after inoculation) with SS19 (three copies per cell), the specific activity of the protein was found to be 487 U/mg. The specific activity of

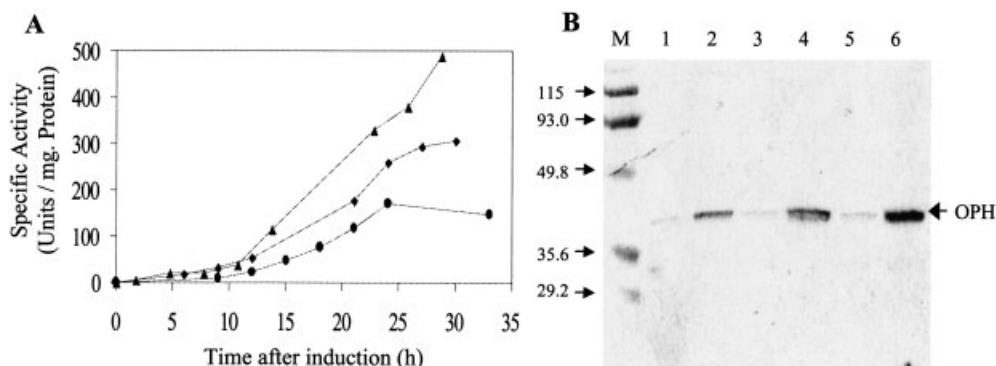


Figure 3. Induction profile for fermentations with strains SS17, SS18, and SS19. (A) Specific activity profiles for the strains containing one (SS17), two (SS18), and three (SS19) chromosomal copies of the *OPH* expression cassette, PPT. Symbols: (●) SS17; (◆) SS18; (▲) SS19. (B) Western blot analysis of fermentation samples. Total cell pellets were diluted to approximately 0.2 g/L DCW and then boiled on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) lysis buffer and resolved on a 12% polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and blotting was done with anti-*OPH* antibody. The figure shows *OPH* expression levels before (approximately 100 g/L dry cell weight; lanes 2, 4, and 6) and after (approximately 130 g/L dry cell weight; lanes 3, 5, and 7) induction for fermentations with strains SS17, SS18, and SS19, respectively. M, broad-range molecular weight marker.

purified OPH (with cobalt as the divalent cation) has been reported to be 8020 U/mg, using the paraoxon enzyme activity assay (Omburo, 1992). As previously noted, the protein content of *R. eutropha* was found to be 68% of dry cell weight (Henderson and Jones, 1997). Thus, the specific activity of 487 U/mg corresponds to 6% of total cellular protein, respectively. The product of 107.3 g/L biomass, 0.68 g protein/g biomass, and 0.06 g OPH/g protein yields an OPH concentration of 4.3 g/L. This is approximately 30 times greater than the levels produced in *E. coli* and 3 times greater than the previously published data in *R. eutropha* (Srinivasan et al., 2002).

CONCLUSIONS

In our earlier work (Srinivasan et al., 2002), we reported the development of a simple fermentation process for producing high levels of recombinant proteins in *R. eutropha*. We showed that plasmids are unstable in *R. eutropha*, even in the presence of antibiotic. We overcame this difficulty by integrating multiple copies of our gene of interest into the chromosome and thereby increasing the gene dosage while eliminating instability. In *E. coli*, translational fusions were made with OPH to increase the specific activity of the expressed protein and to reduce inclusion-body formation (Wu et al. 2001).

Our study with *R. eutropha* has shown that a linear increase in OPH production can be achieved by integrating multiple copies of the gene of interest into the chromosome. Moreover, we have shown that the basal level of induction is not increased substantially, and that multiple copies remain stable in the chromosome. It should be noted that no inclusion bodies were seen, even with such high-level expression of the gene of interest. The levels of protein achieved with three-copy integrant (4.3 g/L) is the highest reported for this protein. We also believe that gene dosage can be further increased by integrating more copies, but it may increase basal expression levels.

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